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ACUTE TOXICITY OF THE CATIONIC SURFACTANT C12-BENZALKONIUM IN DIFFERENT BIOASSAYS: HOW TEST DESIGN AFFECTS BIOAVAILABILITY AND EFFECT CONCENTRATIONS

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Abstract: Using an ion-exchange–based solid-phase microextraction (SPME) method, the freely dissolved concentrations of C12-benzalkonium were measured in different toxicity assays, including 1) immobilization of *Daphnia magna* in the presence or absence of dissolved humic acid; 2) mortality of *Lumbriculus variegatus* in the presence or absence of a suspension of Organisation for Economic Co-Operation and Development (OECD) sediment; 3) photosystem II inhibition of green algae *Chlorella vulgaris*; and 4) viability of in vitro rainbow trout gill cell line (RTgill-W1) in the presence or absence of serum proteins. Furthermore, the loss from chemical adsorption to the different test vessels used in these tests was also determined. The C12-benzalkonium sorption isotherms to the different sorbent phases were established as well. Our results show that the freely dissolved concentration is a better indicator of the actual exposure concentration than the nominal or total concentration in most test assays. *Daphnia* was the most sensitive species to C12-benzalkonium. The acute *Daphnia* and *Lumbriculus* tests both showed no enhanced toxicity from possible ingestion of sorbed C12-benzalkonium in comparison with water-only exposure, which is in accordance with the equilibrium partitioning theory. Moreover, the present study demonstrates that commonly used sorbent phases can strongly affect bioavailability and observed effect concentrations for C12-benzalkonium. Even stronger effects of decreased actual exposure concentrations resulting from sorption to test vessels, cells, and sorbent phases can be expected for more hydrophobic cationic surfactants. *Environ Toxicol Chem* 2014;33:606–615. © 2013 SETAC

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INTRODUCTION

Surfactants are commercially important organic chemicals. Permanently charged quaternary ammonium compounds (QACs), such as benzalkonium and cetrimonium salt, are used as antimicrobial and disinfectant agents [1]. Quaternary ammonium compounds have the potential to disrupt cell membranes [2]. The acute toxic effects of cationic surfactants in different assays have been studied and summarized in scientific reviews [1,3–5]. Toxicity data for QACs and other cationic surfactants are available for algae [6–10], invertebrates [7,11–14], fish [3,7], and mesocosms [15]. Quaternary ammonium compounds and other cationic surfactants are challenging test substances to study. The combination of hydrophobic alkyl chains and positively charged head groups favors adsorption to a multitude of (negatively charged) surfaces. Strong losses from adsorption to glassware; dissolved organic matter in freshwater tests; or proteins in serum of in vitro assays, pipette tips, or the test organisms themselves may render the actual dissolved test concentrations in toxicity tests much lower than the intended (and often reported) nominal concentrations. It can, therefore, be questioned whether reported (nominal) effect concentrations adequately reflect the intrinsic toxicity of the tested cationic surfactants. Adequate risk assessment of surfactants is further complicated, because no common samplers are widely available to detect freely dissolved concentrations for QACs, which are generally considered to drive the toxicity of organic chemicals [16]; many surfactants are present as technical mixtures, the composition of which may change as a result of adsorption or other processes; and the influence of specific ionic interactions among surfactants and environmental substrates and organismal target sites are not well understood. Basic physicochemical properties such as octanol–water partition coefficients are not available or are irrelevant for extrapolation of sorptive properties because, for example, octanol is a neutral solvent that does not account for ionic interactions that occur with many environmental substrates.

In risk assessment of organic contaminants, the equilibrium partitioning theory plays an important role. According to Di Toro et al. [17], effect concentrations in sediment can be predicted from concentrations on a porewater basis and a sediment–water sorption coefficient. Effect data from water-only exposures can then be applied to predict toxicity in sediment. Understanding sorption affinities to relevant sorbent phases or measuring freely dissolved porewater concentrations is thus essential for sediment risk assessment based on equilibrium partitioning. For systems in which the organic chemical has reached equilibrium among sediment, porewater, and organism, the equilibrium partitioning theory [17] assumes the same effective exposure concentrations regardless of exposure route (i.e., uptake of freely dissolved compounds in porewater via skin or gills or uptake via ingestion of contaminated particles). Jager et al. [18] experimentally demonstrated that, for earthworms exposed to a range of hydrophobic nonpolar organic compounds, uptake via ingestion

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may result in faster uptake than uptake from porewater via the skin for very hydrophobic compounds, but also that eventually comparable tissue concentrations are reached. This equilibrium partitioning can also easily be applied to experiments in water-only tests in the presence of a dissolved matrix (instead of particulate matter as in sediment) that may bind the compound. In the presence of binding matrices, the concentration of the unbound chemical (i.e., the freely dissolved concentration) is the relevant dose parameter. Several studies in the field of ecotoxicology, but also in the area of in vitro toxicology, have shown that the freely dissolved concentration represents a more intrinsic dose parameter for analyzing toxicity data [16]. This equilibrium partitioning concept has been shown to be relevant for nonpolar hydrophobic contaminants, but the validity of this concept for ionic compounds has hardly been studied. Recently, the concept was applied in toxicity studies with anionic and nonionic surfactants [19,20]. Within the equilibrium partitioning approach, effect concentrations are often estimated using sorption and accumulation models. However, the actual measurement of freely dissolved concentrations in soil or sediment tests or in vitro cell-based assays represents an even more powerful tool [21–24].

In our recent work, passive samplers (solid-phase micro-extraction [SPME]) were developed to analyze freely dissolved concentrations for QAC surfactants [25]. This SPME technique was also used to determine freely dissolved concentrations in humic acid (HA) solutions to determine sorption affinity under varying pH and salinity conditions [26]. Similar samplers were applied in sediment toxicity tests for neutral and anionic surfactants in toxicity studies [19,20]. These studies showed that the equilibrium partitioning [17] is also applicable to both nonionic and anionic surfactants; that is, the sediment toxicity expressed as the concentration in sediment porewater is equal to the toxicity measured in water-only exposure [19,20]. Another study from Van Wijk et al. [6] showed that effect concentrations for algae exposed to QACs were decreased in the presence of clay minerals, sediment, or HAs, indicating the strong effect of sorption on bioavailability. Thomas et al. [12] showed that the acute toxicity of the QAC didecyldimethylammonium bromide to the soil-dwelling nematode *Caenorhabditis elegans* decreased with increasing cation-exchange capacities (CEC) of soils, and a loading of up to 15% of the CEC was required to induce an effect in most soils. These toxicity studies on QACs indicate that the equilibrium partitioning applies also to QACs and that bioavailability of QAC can be strongly limited by adsorption. However, no measurements of freely dissolved concentrations were performed to verify that freely dissolved concentrations at effect levels are constant.

The aims of the present study were to verify the equilibrium partitioning for QACs in acute toxicity tests with 4 test organisms that are commonly used in bioassays and to assess the influence of sorptive processes on toxicity in such assays by performing detailed mass balance analyses. Applying the newly developed sampling tools for QACs [25], we sought to measure the freely dissolved and total concentrations of the QAC C12-benzalkonium in different toxicity assays, including 1) immobilization of *Daphnia magna* in the presence and absence of dissolved HA; 2) mortality of *L. variegatus* in the presence and absence of a suspension of Organisation for Economic Co-Operation and Development (OECD) sediment; 3) photosystem II inhibition of green algae *Chlorella vulgaris*; and 4) viability of in vitro rainbow trout gill cell line (RTgill-W1) in the presence and absence of serum proteins. We specifically focused on the difference between effect concentrations based on nominal concentrations (total spiked amount in the medium) and those based on measurements of total dissolved concentrations and freely dissolved concentrations. The study also determined the loss from chemical adsorption to the different test vessels used in these tests. Our previous work showed a strong affinity of QACs for natural HAs [25], so we included other types of sorbent phases that are commonly used in toxicity studies, such as serum protein, artificial OECD sediment, and algal cells. Whereas the effect of QAC adsorption to walls of test vessels applies to all test systems, additional decreases of actual dissolved concentrations may occur in in vitro cell assays in which the medium also contains serum or serum protein to which chemicals may bind. Bioassays using freshwater instead of synthetic medium may also have HAs present in the medium. Oligochaete worms exposed to sediment may be exposed to chemicals via ingested sediment, as suggested for the toxicity of the QAC dimethyldioctadecylammonium chloride (DODMAC) to the oligochaete worm *Lumbriculus variegatus* [11]. Sorbed fractions are not taken up by cell systems such as algae and cells cultured in vitro, but interesting test cases for validation of the equilibrium partitioning theory are filter-feeding daphnids that may ingest surfactant sorbed to HA [27] and the oligochaete worm *L. variegatus* that can ingest sediment particles.

MATERIALS AND METHODS

Chemicals, materials, and analysis

The QAC applied in the bioassays was benzyldimethyldodecyl-ammonium chloride (C12-BAC; ≥99.0%) obtained from Sigma-Aldrich. Hexadecyltrimethylammonium-d42 chloride (CDNisotope) served as internal standard for analysis by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Table 1 provides an overview of the media, sorbents, and test vessels used in the different bioassays. Humic sodium salt (catalog number. H1675-2; Aldrich HA [AHA]) used in the *Daphnia* assay was obtained from Sigma-Aldrich. The OECD sediment was made according to OECD guideline 225, containing 74.5% quartz sand (Sigma-Aldrich), 5% finely ground sphagnum peat as organic matter (garden centre Intratuin), and 20% kaolinite clay (Imerys Cornwall) [28]. The cyanobacteria BG-11 freshwater medium (50 times diluted with Millipore water for algal culture) was purchased from Sigma-Aldrich. Leibovitz's L-15 medium, fetal bovine serum (FBS), penicillin, and streptomycin, used to culture RTgill-W1 cells, were purchased from Invitrogen Life Technologies. Sodium dodecyl sulfate (SDS), used as a positive control in the in vitro assay, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Toxicity exposure media, including Dutch standard water (DSW) [29], L-15/ex (L-15 medium constituents without vitamins, amino acids, and glutamine) [30], were prepared according to standard procedures in Millipore water from a Milli-Q purification system. Disposable 7- and 30-mm polyacrylate (PA)-coated SPME fiber (Polymicro Technologies) was used throughout the sorption and toxicity experiments.

Sorption test

The setup for C12-BAC sorption experiments to algal cells (3.7×10^7 cells/mL in BG-11 medium, manually counted with a Bürker counting chamber) and protein (10 g/L BSA or 10% FBS) was similar to the one described previously with AHA [25]. To attain the desired freely dissolved concentrations prior to the toxicity tests, the sorption affinities of C12-BAC to AHA and OECD sediment in DSW were estimated from other sorption experiments performed in different matrix. The sorption tests of

Table 1. Bioassay properties

Bioassay species	Medium	Sorbent	Exposure (h)	End points	SPME fiber	Test vessel
Daphnid *Daphnia magna*	DSW	AHA	48	Imobilization	7-μm PA	Glass beaker 50 mL
Worm *Lumbriculus variegatus*	DSW	OECD sediment[b]	48	Imobilization	7-μm PA	Scintillation glass vial 20 mL
Freshwater algae *Chlorella vulgaris*	BG-11[a]	Not applicable	22	Φ_{PSII} efficiency	35-μm PA	Polystyrene 12-well Plate 2 mL
Rainbow trout gill cell line RTgill-W1	L-15/ex	FBS/BSA	24	Cell viability	35-μm PA	Polystyrene 24- well Plate 5 mL

[a]Fifty times diluted BG-11 medium with Millipore water.
[b]The composition of the OECD sediment shown in Supplemental Data, Table S1, and prepared according to OECD guideline 225 [28].
SPME = solid-phase microextraction; DSW = Dutch stand water; AHA = Aldrich humic acid; OECD = Organisation for Economic Co-Operation and Development; L-15/ex = L-15 medium constituents without vitamins, amino acids and glutamine; BSA = bovine serum albumin (10 g/L); FBS = fetal bovine serum (10%); PA = polyacrylate; Φ_{PSII} = photosystem II efficiency.

C12-BAC to AHA (10 mg/L in 5 mM Na^+ at pH 6) and OECD sediment (1.25 g/L in 15 mM Na^+ at pH 6) were conducted separately according to the previous setup [25], which was slightly different from the amounts applied in the toxicity test (20 mg/L AHA in DSW at pH 8.1 and 5 g/L in DSW at pH 8.2). Therefore, the sorption isotherms for AHA, sediment, and protein were generated from the toxicity tests. Generally, an aliquot of surfactant stock in methanol was spiked in the test solution with the sorbing phase and allowed 24 h (1 h for protein medium) for equilibration in a climate room (20 °C ± 1 °C). In the 24-mL solution with sediment, AHA, or algae, a single 3-cm piece of 7-μm polyacrylate (PA)-coated SPME fiber was used to measure freely dissolved surfactant concentration. To satisfy the condition of negligible depletion of the dissolved concentration by the SPME measurement, a single 1.7-cm piece of the 35-μm PA-SPME fiber was used in the 2-mL protein medium, because C12-BAC showed a substantially lower affinity for that fiber. The C12-BAC sorptions to the 2 types of PA-SPME fiber were calibrated in the same medium with the same exposure period as applied in the sorption and toxicity experiments, including measurements of aqueous concentrations by solid-phase extraction columns (WCX-SPE; Waters) [25]. Linear SPME calibration curves could be fitted in the low concentration range, whereas a polynomial equation was used for the highest concentrations. SPME fibers were always wiped with wet tissue after extraction from the test medium, to avoid contaminating the desorption solution with HA, sediment particles, algae, or proteins.

Different bioassays in the toxicity test

Water flea D. magna. *Daphnia magna* was taken from a stock cultured in M4 Elendt medium at 20.5 °C, fed with *Chlorella vulgaris*. The test was performed according to OECD guideline 202 [31]. We used 50-mL glass beakers as test vessels, each containing 50 mL of DSW or DSW-AHA solution (7 mg/L and 20 mg/L, respectively), all at pH 8.1. Beakers were covered by glass plates during the test. Experiments were carried out in a temperature-controlled room (20 ± 2 °C) with a 16:8-h light: dark regime. Neonates less than 24 h old were used and were obtained from *Daphnia* aged 2 to 4 wk. Experiments were performed in duplicate (parallel) series of 5 test concentrations with 5 animals exposed. The control was composed of 20 animals divided into 4 batches. The test was performed as a static test for 48 h. Those animals that were not able to swim within 15 s after gentle agitation of the test vessel were considered to be immobile and were recorded. The tests were inspected at 24 h and 48 h. Seven-micrometer PASPME fibers were exposed in duplicate in each beaker for 3 h to determine the free concentration at the beginning and end of the test. Concentrations from the 4 SPME fibers at each concentration were averaged, and freely dissolved concentrations were calculated from a calibration curve derived in the same test medium with 3 h exposure.

Oligochaete worm L. variegatus. *Lumbriculus variegatus* was cultured in flow-through aquaria containing tap water and cellulose substrate at a temperature of 23 ± 1 °C. Before testing, organisms were left overnight under gently running tap water to empty their guts. The DSW for worm tests was aerated continuously for 24 h. Approximately 100 mg sediment was first added to each glass vial, which was then filled with 24 mL DSW. Experiments were performed in duplicate. A series of 5 concentrations was tested, and the solutions were spiked with a stock in methanol (<1% methanol). Ten worms were carefully transferred via a plastic pipette to each vial. At 48 h, the surviving worms in each test vial with sediment were counted. For water-only systems, pilot tests showed that it was not feasible to maintain steady exposure concentrations with 10 worms in each vial (see *Discussion*). Therefore, worms were exposed individually to 5 concentrations in 24 mL DSW in triplicate. Worm survival in water-only systems was counted daily during 9 d. Blank controls (spiked with 100 μL methanol) were prepared for both DSW-sediment (2 vials, each with 10 worms) and DSW-only systems (5 vials, each with 1 worm). Single 3-cm pieces of 7-μm SPME fibers were exposed for 24 h in each vial starting at 48 h. SPE samples of the total dissolved concentrations were taken at 48 h as well.

Green algae C. vulgaris. *Chlorella vulgaris* (Beyerinck UTEX 259) was cultured in BG-11 medium at 16 °C under a 16:8-h light:dark regime with a light intensity of 50 μE (F58W/BriteGro 2084). The initial algal cell density, counted manually with a Bürker counting chamber, was 3.1×10^6 cells/mL for the toxicity test. Five milliliters of algae suspension was then pipetted to each well in a 12-well polystyrene plate (Cellstar; Greiner Bio-One). Twenty microliters of surfactant stock with different concentrations in methanol was spiked in the well plate.

We first performed a rang-finding test using 5 concentrations of the test chemical, with duplicate wells per concentration. Algal medium with the spiked chemical was well mixed with a 1-mL polyethylene pipette tip (also to equilibrate the chemicals on the tip). Then, 2 replicates of 280-μL samples were pipetted in a black polypropylene 96-well plate (Greiner Bio-One). Pulse amplitude modulation (PAM) fluorometry (Water-PAM), based on chlorophyll *a* fluorescence, was used to determine the effective photosystem II efficiency (Φ_{PSII}) [32], which was measured at 1 h, 4 h, 22 h, and 48 h. A single 3–cm, 35-μm PA fiber was exposed for 1 h in each 12-well plate after each sampling time point to verify that sorption to algae and polystyrene well was significant. The whole algal suspension was extracted via SPE at the end of the test to verify total surfactant concentrations in the medium. Sorption to the polystyrene wall was further checked via extracting the empty well with 5 mL SPE eluent for 30 min. A second experiment using

5 concentrations in a smaller range around the effect concentration was performed in the same way as the range-finding test, except that toxicity was determined by PAM only at 22 h.

In vitro RTgill-W1 cells. The rainbow trout gill epithelial cell line RTgill-W1 was purchased from the American Type Culture Collection (CCL-163). The protocols for the cytotoxicity assays have been well described in the literature [33,34]. Cells were cultured at 20 °C in the dark and in sealed culture flasks with Leibovitz's L-15 culture medium supplemented with 100 U/L penicillin, 100 μg/L streptomycin, and 10% FBS (containing 40 mg/mL BSA, as communicated by the supplier), based on the procedures of an earlier study [33]. Experiments were performed at passages 6 to 38 under sterile condition. Cells were seeded at a density of 1.2×10^5 cells/mL. Subsequently, 2 mL of medium was transferred to each well in a 24-well plate (Cellstar; Greiner Bio-One). After incubation at 20 °C for 24 h, culture medium in each well was removed, and the wells were filled with 2 mL of exposure medium. For the medium-only test, each well was filled with L-15/ex medium and spiked with 10 μL stock of the chemical to give 6 concentrations in triplicate. For the protein-medium test, 10% FBS prepared in L-15/ex medium was spiked in triplicate with 6 surfactant stocks and then well mixed, equilibrating for 1 h. Afterward, the spiked exposure medium was transferred to wells with cells. Similar test series were run with medium containing 10 g/L (~0.15 mM) BSA. Cells were exposed for 24 h in the dark at 20 °C for both series of tests. Blank controls were prepared with 0.5% methanol in triplicate for each test. Six concentrations of SDS in triplicate served as positive controls for passages 8 and 30 to check the reliability of the assay performance. Single 35-μm PA fiber served as the sampling tool for measuring freely dissolved surfactant based on 1-h exposure starting at 23 h.

To determine QAC sorption to the polystyrene walls, 2 concentrations of C12-BAC (0.15 and 5 mg/L) were prepared separately in the L-15/ex medium with and without 10% FBS solution and analyzed at 5 min, 1 h, and 24 h. For the medium containing cells, the sorption to cells was measured by first detaching cells from the well bottom with 100 μL trypsin and then extracting total suspensions with SPE at 24 h. The well was then further extracted with SPE eluent to determine sorption to polystyrene, similarly to the algae test.

Analysis

C12-BAC was measured using a LC-MS/MS system (MDS Sciex API 3000 LC-MS/MS System; Applied Biosystems), as described in detail previously [25]. Chromatograms were quantified in Analyst 1.4.2 (MDS Sciex Applied Biosystems). Experimental data were analyzed in GraphPad Prism 6.01.

RESULTS

Water flea D. magna

The 48-h exposure–response curves of C12-BAC to *D. magna* in the presence and absence of AHA are shown in Figure 1A. Based on total spiked surfactant concentrations (SPE columns, $C_{W,total}$), median effect concentrations (EC50s) are a factor 5 higher for the 20 mg/L AHA solution compared with the water-only test (Table 2). Based on freely dissolved concentrations via SPME measurements (SPME fiber, $C_{W,free}$), the exposure–response curves for the tests performed in 7 and 20 mg/L AHA solutions are not significantly different from the water-only test (analysis of variance, $p = 0.28$), and EC50 values based on freely dissolved concentrations ($EC50_{free}$) are constant at 0.016 mg/L (standard error [SE] = 0.003, $n = 3$; Table 2). The fitted $EC50_{free}$ and $EC50_{nominal}$ (EC50 based on nominal concentrations) values as a function of AHA concentration are presented in Figure 2. This $EC50_{free}$ value (0.016 mg/L) in the water-only test is 30% lower than the $EC50_{nominal}$ (0.023 mg/L). This difference suggests that there is some loss of the compound in the test system, most likely from sorption to the glass beaker. The data for the distribution of the chemical in AHA solution (Figure 1B) are based on the assumption of a 100% mass balance. This assumption is plausible because a previous study indicated that recovery percentages above 90% could be attained for C12-BAC–spiked AHA solution [25]. The sorption isotherm of C12-BAC to 20 mg/L AHA in 50 mL DSW is also calculated based on the assumption of 100% mass balance (Figure 1C). The sorption coefficient to AHA of 5.4 log units in this toxicity test is comparable to the value reported in our earlier sorption study with C12-BAC [25]. The influence of AHA on the effect of C12-BAC on immobilization of *D. magna* shows that AHA reduces the bioavailability of C12-BAC and that toxicity is directly related to the freely dissolved concentration.

Oligochaete worm L. variegatus

In a water-only pilot study with 10 worms in each test vial, $C_{W,free}$ and $C_{W,total}$ measured in the beginning of the test were comparable to the $C_{W,total,48\,h}$ in the system (see Supplemental Data, Figure S1). However, we detected a strongly decreased $C_{W,free}$, after 48 h in vials in which several worms had died, whereas $C_{W,total}$ after 48 h remained constant. The full recovery of the SPE extracts indicated that there was no microbial degradation of the surfactant. We assume that this strong decrease in freely dissolved concentration was related to

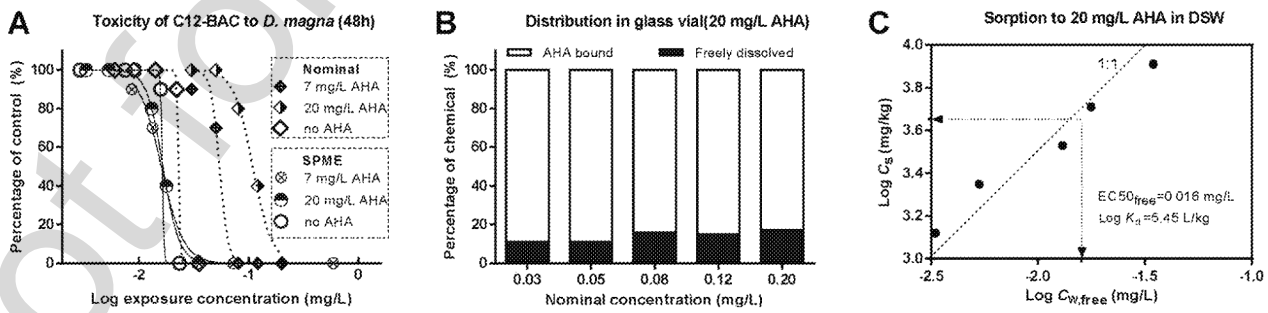

Figure 1. Exposure–response curve of benzyldimethyldodecyl-ammonium chloride (C12-BAC; ≥99.0%) to *Daphnia magna* for 48 h in 50 mL Dutch standard water (DSW) with and without 7 mg/L or 20 mg/L Aldrich humic acid (AHA) in a glass beaker. (**A**) Immobilization of the organism, expressed as a percentage of control, is plotted against both nominal and free concentrations (solid-phase microextraction). Distribution (**B**) and sorption isotherm (**C**) of C12-BAC in 50 mL DSW with 20 mg/L AHA system, assuming 100% of mass balance. Dashed line indicates 1:1 relationship, and logarithmic sorption coefficient (K_d) is calculated at the median effect concentration value based on the measurement of free concentration ($EC50_{free}$). C_s = AHA sorbed concentration; $C_{w,free}$ = freely dissolved concentration.

Table 2. Effect concentrations for benzyldimethyldodecyl-ammonium chloride (≥99.0%)

Bioassay species	Sorbent phase	Medium-only $EC50_{free/total}$ (95% CI)[a] (mg/L)	Sorbent-phase $EC50_{free}$ (95% CI)[a] (mg/L)	Sorbent-phase $EC50_{nominal}$ (95% CI)[a] (mg/L)	Sorbent- phase log K_d at $EC50_{free}$ (L/kg)
Daphnia magna	7 mg/L AHA	0.016[b]	0.016 (0.011–0.024)[c]	0.054 (0.039–0.075)[c]	5.45[d]
	20 mg/L AHA		0.016 (0.015–0.018)[d]	0.109 (0.096–0.123)[d]	
Lumbriculus variegatus[h]	5 g/L OECD sediment	1.62[b]	0.57[b]	12.06[b]	3.68
Chlorella vulgaris	3.1×10^6 algae	5.80 (4.99–6.75)	Not applicable	Not applicable	Not applicable
Rainbow trout gill cell line-W1	10% FBS or	0.31 (0.27–0.36)	0.21 (0.17–0.28)[e]	1.10 (0.97–1.22)[e]	1.62[g]
	10 g/L BSA		0.18 (0.15–0.21)[f]	0.83 (0.76–0.91)[f]	2.98

[a]EC50 values based on free (SPME), total (SPE) or nominal concentration with 95% confidence interval (CI).
[b]95% CI cannot be determined.
[c]Data for 7 mg/L AHA solution.
[d]Data for 20 mg/L AHA solution.
[e]Data for 10% FBS.
[f]Data for 20 mg/L 10 g/L BSA.
[g]The unit of log K_d is expressed as L/L FBS.
[h]Expressed as LC50 instead of EC50.
$EC50_{free}$ = median effect concentrations based on the measurement of free concentration; $EC50_{nominal}$ = median effect concentrations based on the nominal concentration; AHA = Aldrich humic acid; OECD = Organisation for Economic Co-Operation and Development; DSW = Dutch stand water; BSA = bovine serum albumin; FBS = fetal bovine serum; K_d = sorption coefficient; LC50 = median lethal concentration; SPE = solid-phase extraction; SPME = solid-phase microextraction.

sorption of the surfactants to degraded tissues of dead worms. This was at least partially confirmed by a test with worms that had been killed and preserved intact by 0.5% of formaldehyde prior to exposure, in which we observed no decrease in free or total concentrations within 48 h, suggesting negligible chemical penetration to and sorption into the intact worm. Therefore, we exposed worms individually in water-only tests to prevent the effect of a few dead worms on freely dissolved C12-BAC concentrations. In systems with sediment, pilot tests showed such a strong sorption to sediment that this should buffer any additional decrease of freely dissolved concentrations by degraded worm tissue.

Figure 3A shows the exposure–response curve of C12-BAC to *L. variegatus* in the DSW-only medium and the system with 5 g/L OECD sediment for 48-h exposure. The survival compared with the control is plotted against both $C_{W,nominal}$ and measured $C_{W,free}$. As shown in Table 2, the presence of 5 g/L sediment leads to a difference in median lethal concentration (LC50) values from 12.06 mg/L for nominal concentrations to 0.57 mg/L based on free concentrations, a factor of 21. The

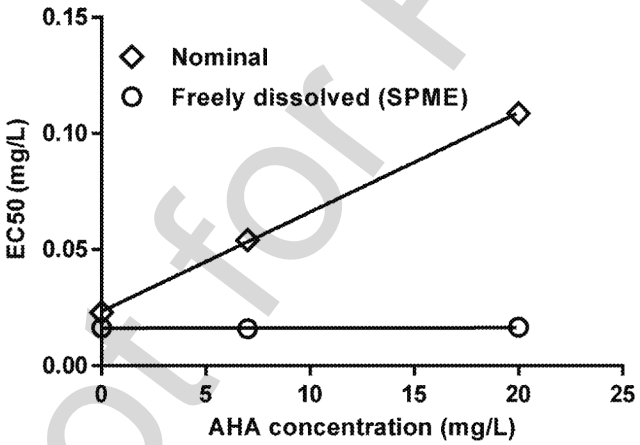

Figure 2. Median effect concentrations (EC50s) of benzyldimethyldodecyl-ammonium chloride (C12-BAC; ≥99.0%) to *Daphnia magna* after 48 h, based on freely dissolved (solid-phase microextraction [SPME]) and nominal concentration, as a function of increasing Aldrich humic acid (AHA) concentration. The linear regression is used to fit both the nominal and SPME data.

fraction of C12-BAC that is freely dissolved or sediment bound is presented in Figure 3B. Only 5% of the chemical is freely dissolved in a 5 g/L sediment system. Adsorption to glass is not a significant contribution to the mass balance at the applied sediment concentration present in the medium. A sorption isotherm to sediment, calculated from these measurements and the assumption of a 100% mass balance, is presented in Figure 3C, which suggests a concentration-independent sorption coefficient of 3.7 (log value). The sorption isotherm for OECD sediment in DSW generated from the toxicity test is also comparable with the sorption isotherm obtained in 15 mM Na^+ at pH 6, as shown in Figure S2. The 48-h $LC50_h$ value measured with SPE in water-only systems is 1.62 mg/L, which is a factor of 3 above the 48-h LC50 measured in sediment systems based on the measured $C_{W,free}$ (0.57 mg/L; Table 2). These water-only 48-h LC50 data for *L. variegatus* are a factor of 30 to 100 higher than the 48h EC50 for *D. magna*. The freely dissolved concentration in the sediment test seems to overpredict toxicity compared with the water-only test, which may imply that there are additional exposure to the worms via ingestion of the sorbed fraction. Figure S3 depicts the exposure–response curves monitored in water-only tests at 7, 30, 48, 72, 96, and 216 h. The LC50 value is constant up to 48 h (average $LC50 = 1.70 \pm 0.2$ mg/L, $n = 3$) but gradually drops to 0.26 mg/L after 9 d of exposure (with full survival of worms in the control exposures after 9 d). The observed difference in 48-h LC50 between water-only exposure and exposure to sediment suspension may therefore be the result of a kinetic effect, in which chemical equilibrium is not yet reached within 48 h and uptake via sediment increased kinetics. However, a detailed analysis of the toxicokinetics is beyond the aim of the present study, and the issue will not be discussed in the present study. There is unfortunately no data for longer exposure to sediment to determine the effect of exposure time on the LC50, which could resolve this issue. Still, the freely dissolved QAC concentration in solids suspensions with worm again appears to be a better dose parameter as a bioavailable concentration than the nominal concentration that includes the sorbed fraction.

Green algae C. vulgaris

Figure 4A shows the effect of C12-BAC on the Φ_{PSII} of *C. vulgaris*. The exposure–response curves based on $C_{W,free}$,

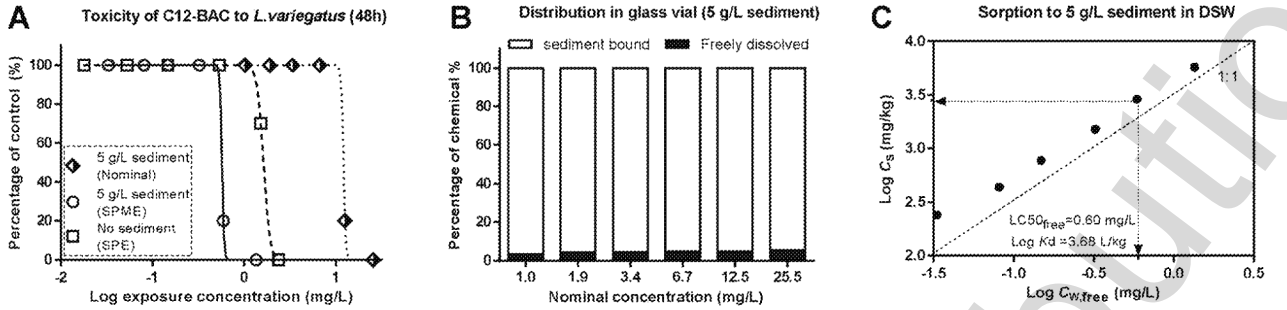

Figure 3. Exposure–response curve of benzyldimethyldodecyl-ammonium chloride (C12-BAC; ≥99.0%) to *Lumbriculus variegatus* for 48 h in 24 mL Dutch standard water (DSW) with and without 5 g/L Organisation for Economic Co-Operation and Development (OECD) sediment in the scintillation glass vial. (**A**) Survival of the organism, expressed as a percentage of control, is plotted against both nominal and measured free (solid-phase microextraction) or total concentration (solid-phase extraction). Distribution (**B**) and sorption isotherm (**C**) of C12-BAC in 24 mL DSW with 5 g/L OECD sediment assuming 100% of mass balance. Dashed line indicates 1:1 relationship, and logarithmic sorption coefficient (K_d) is calculated at the median lethal concentration value based on the measurement of free concentration ($LC50_{free}$). C_s = sediment sorbed concentration; $C_{w,free}$ = freely dissolved concentration.

$C_{W,total}$, and $C_{W,nominal}$ in the algae assay overlap. The data are combined with 2 series of tests that provide virtually the same EC50 outcome. The mass balance data presented in Figure 4B indicate that sorption of C12-BAC to *C. vulgaris* at a density of 3.1×10^6 cells/mL is not significant. The data based on the SPME measurements were combined from the 2 test series, resulting in the 22-h EC50 value of 5.8 mg/L, 5-fold and 300-fold higher than the 48-h EC50 for worm and *Daphnia*, respectively (Table 2). The exposure–response curve for 1 h, 4 h, 22 h, and 48 h based on the measured $C_{W,total}$ at 48 h, as shown in Figure S4, suggests that the toxic effect concentration decreased between 1 h and 4 h but was relatively stable between 4 h and 48 h of exposure.

The chemical distribution in the test system depicted in Figure 4B demonstrates that chemical sorption to the 5-mL polystyrene well is concentration dependent. In this algal assay, 72%, 39%, and 10% of the total spiked chemical are bound to the polystyrene wall at a nominal concentration of 0.02 mg/L, 0.41 mg/L, and 2.04 mg/L, respectively. At $C_{W,nominal}$ greater than 10 mg/L, the losses to the polystyrene 12-well plate system are negligible, which explains the overlap between $EC50_{nominal}$ and $EC50_{free}$ at the EC50 level of 5.8 mg/L. A relatively low recovery, 79% and 77% for the sample of 0.02 mg/L and 0.41 mg/L, respectively, was observed, probably because the washing step prior to wall extraction flushed out some polystyrene-bound chemical before the well extraction or repeated pipetting caused some loss. For the other 3 high-concentration samples, more than 90% of the chemical in the system was recovered. To study the sorption to algae, another batch sorption experiment was performed at a cell density 12 times higher, at 3.7×10^7 cells/mL in diluted BG-11 medium. The sorption isotherm displayed in Figure 4C indicates a strongly nonlinear sorption behavior with a Freundlich exponent (n_F) of 0.25. The algae–water distribution coefficient of 400 L/kg dry weight at 1 mg/L confirms that sorption to algae at effect concentrations of 5 mg/L has a negligible effect on toxicity (and is much lower than the sorption affinity to AHA). Still, sorption can be a substantial mass balance fraction at sublethal doses (6.3×10^4 at 1 μg/L). Also, Van Wijk et al. [6] observed strong and nonlinear sorption of the QAC hexadecyltrimethylammonium to the freshwater alga *Pseudokirchneriella subcapitata* (n_F 0.39) but at a much higher concentration range (>10 mg/L) than used in the present study.

In vitro RTgill-W1 cells

Cytotoxicity was assessed by exposing C12-BAC to RTgill-W1 cells for 24 h. The viability was measured using the Alamar blue assay and expressed as a percentage of control. Sodium dodecyl sulfate positive control EC50s were stable at a range of 12.4 to 14. mg/L ($n = 2$), indicating constant cell performance for all passages. Figure 5A depicts the exposure–response curves of C12-BAC to RTgill-W1 with and without 10% fetal bovine serum (FBS) solution as a function of measured $C_{W,free}$ and

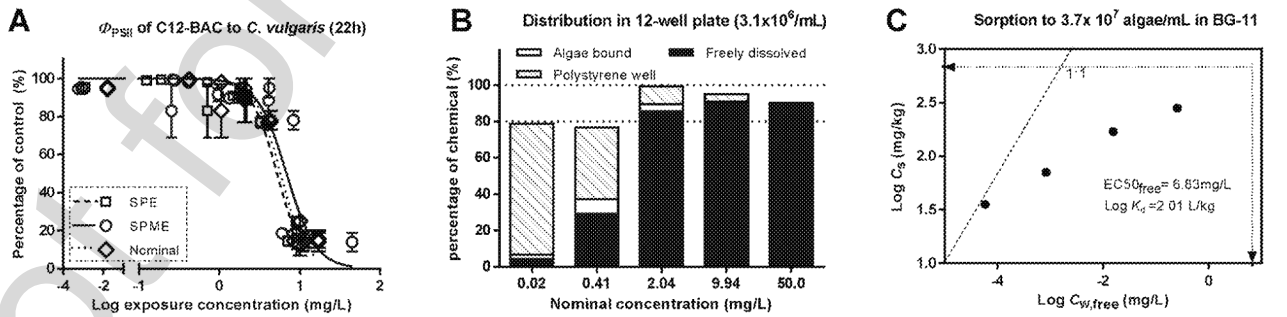

Figure 4. Exposure–response curve of benzyldimethyldodecyl-ammonium chloride (C12-BAC; ≥99.0%) to *Chlorella vulgaris* in 22 h at a density of 3.1×10^6 cells/mL in 5 mL BG-11 medium in the polystyrene 12-well plate. (**A**) Photosystem II efficiency (Φ_{PSII}) of *C. vulgaris*, expressed as a percentage of control (± standard deviation), is plotted against both nominal and measured free (solid-phase microextraction) or total concentration (solid-phase extraction); (**B**) distribution of C12-BAC in the with *C. vulgaris* (3.1×10^6 cells/mL) in the test system; (**C**) sorption isotherm of C12-BAC to *C. vulgaris* at a higher density of 3.7×10^7 cells/mL in BG-11 medium. Dashed line indicates 1:1 relationship, and logarithmic sorption coefficient (K_d) is calculated at the median effect concentrations based on the measurement of free concentration ($EC50_{free}$). C_s = algae sorbed concentration; $C_{w,free}$ = freely dissolved concentration.

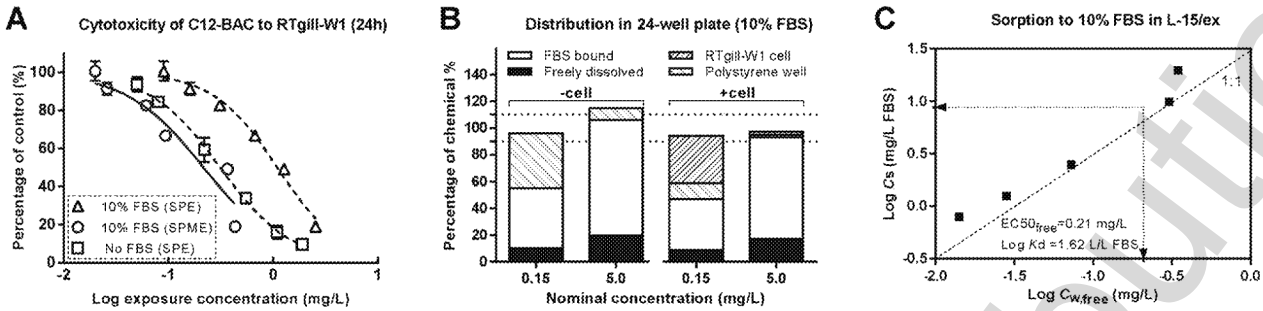

Figure 5. Exposure–response curve of benzyldimethyldodecyl-ammonium chloride (C12-BAC; ≥99.0%) to rainbow trout gill cell line-W1 for 24 h in 2 mL L-15/ex medium with and without 10% fetal bovine serum (FBS) in the polystyrene 24-well plate. (**A**) Viability, expressed as a percentage of control (± standard deviation), is plotted against both measured free (solid-phase microextraction) and total concentration (solid-phase extraction); (**B**) distribution of C12-BAC in 2 mL L-15/ex medium containing 10% FBS in the presence and absence of cell at 2 concentration levels of exposure; (**C**) sorption isotherm of C12-BAC in 2 mL L-15/ex medium containing 10% FBS. Dashed line indicates 1:1 relationship, and logarithmic sorption coefficient (K_d) is calculated at the median effect concentration based on the measurement of free concentration ($EC50_{free}$). C_s = FBS sorbed concentration; $C_{w,free}$ = freely dissolved concentration.

$C_{W,total}$ concentration at 24 h. The EC50 values are presented in Table 2. The presence of 10% FBS solution led to a 3.5 times reduction in free concentration compared with the test in the absence of FBS (based on measured $C_{W,total}$). Moreover, the EC50 values based on $C_{W,free}$ in both the presence and the absence of FBS are not significantly different (F test, $p = 0.74$; see Table 2). This demonstrates that the freely dissolved QAC concentration represents the bioavailable concentration in this type of cell assay. Similar exposure–response curves, EC50 values, and sorption data are presented in Supplemental Data, Figure S5A and B, using 10 g/L bovine serum albumin (BSA, 0.15 mM), the main protein component of FBS. At the highest test concentrations, no cells were observed by visual inspection after 24 h, suggesting that Alamar blue indicated cell viability and not specifically an effect on mitochondrial metabolic activity [30].

The distribution of C12-BAC in the culture medium with and without RTgill-W1 cells was determined at the end of the 24-h exposure (Figure 5B). More than 90% of the chemical was recovered from the test system in the 24-well plate. The chemical sorption to the polystyrene well, when no cell is present on the bottom of the plate, was again (as with the 12-well plate used for *C. vulgaris*) found to be concentration dependent and significant at a $C_{W,nominal}$ of 0.15 mg/L. Such a loss accounts for 43% of the spiked chemical and is almost equal to the fraction of surfactant bound to the 10% FBS (47%). These results illustrate that using $C_{W,nominal}$ as a dose parameter could significantly underestimate the toxicity of QACs (e.g., C12-BAC) even if there is no dissolved sorbent present, because the chemical may also adsorb to the wall of the test system. Only 8% sorbed to polystyrene in the 5.0 mg/L sample without cells. The fraction sorbed to the well in the presence of cells is, however, greatly reduced compared with that in the absence of cell; even at a concentration of 0.15 mg/L, only 13% loss to the wall was observed. On the other hand, 35% of the spiked chemical was recovered via cell extraction, suggesting that the fraction sorbed to cells can be significant. These results also indicate that the sorption area of the polystyrene surface was reduced because the well bottom was effectively covered by the cultured cell layer. Figure 5B shows no cell-bound fraction at a concentration of 5 mg/L, because all cells had detached from the well plate bottom as a result of cytotoxicity. The percentage of chemical bound to FBS ranged 47% to 90% in the culture medium with 10% FBS. Based on the data for the distribution, also a sorption isotherm was established for C12-BAC on FBS dry weight material (Figure 5C). The isotherm is nearly linear with a relatively low sorption affinity of 44 L/L 10% FBS solution at 0.1 mg/L. As shown in Figure S5A, a linear sorption isotherm was also observed for BSA, representative for a major component of serum, but with a sorption coefficient of 869 L/kg BSA at 0.1 mg/L.

DISCUSSION

Sorptive processes of C12-BAC in different assays

Toxicity of QACs has been reported in different bioassays, including invertebrates [13,35], crustaceans [7,12], algae [6,8–10,14], and cell cultures [36–38]. In most studies, the effective doses are often expressed as calculated nominal concentrations or measured total concentrations in the assays. This is not appropriate in some cases, however, because specific and nonspecific sorption processes may reduce the concentration available for uptake [16,39]. For instance, chemicals can specifically bind to organic matter or clay fractions in the sediment [12,39]. Moreover, in the in vitro systems, chemicals may sorb to serum proteins or components in the medium but also bind in significant fractions to the cells [40–42]. Additional losses can still occur if the chemicals are able to sorb to test vessels [14,40,41,43,44]. However, these exposure issues are not often taken into consideration in the interpretation of toxicity test results [45].

In contrast, the present study has provided detailed information on the distribution of C12-BAC in 4 bioassays with different test procedures and toxic endpoints. Not only were the freely dissolved QAC concentrations measured in toxicity tests using SPME for the first time, but the present study is also the first to determine the fractions partitioning to the compartments of the test systems and to investigate the relative contribution of each sorption process.

A dissolved organic carbon concentration of 20 mg/L in the daphnid test reduced the freely dissolved C12-BAC fraction by 80% (Figure 1B). The worm test indicates that 5 g/L OECD sediment was needed to reduce the $C_{W,free}$ by 90% (Figure 3B), indicating a lower sorption coefficient than for AHA. The bioavailable porewater concentrations strongly depend on the sorptive properties of the surfactant to each specific phase. For unknown reasons, sorption of C12-BAC to algae (Figure 4B) and serum material (FBS in Figure 5B or BSA in Supplemental Data, Figure S5A) appears to be orders of magnitude weaker than to AHA. Still, the high serum/BSA amounts typically applied in in vitro studies clearly impact the bioavailability of QACs.

In addition to the binding to suspended solids in the medium, bioavailability of C12-BAC is related to the test equipment.

Figure 2 shows a small offset between $EC50_{free}$ measured by either SPME or nominal concentrations, suggesting a small influence of QAC binding to the glass vessels. Figures 4B and 5B show the concentration-dependent adsorption of the chemical on polystyrene well plates. Polystyrene sorption did not influence the determination of EC50 values in tests with algae and RTgill-W1 cells. Using the nominal concentration as the dose parameter may overestimate the level at which toxicity starts to occur, and toxicity tests with more sensitive species may be strongly influenced by binding to the polystyrene wells.

Sorption to intact alive or dead worms was negligible, whereas decomposed worm tissues appeared to strongly influence freely dissolved concentrations. This further suggests that internal worm tissues might not act as sorption sites for QACs during the acute exposure period. Sorption to algae in our test setup was negligible, but this may clearly depend on algal densities as well as algal species. Figure 5B and Supplemental Data, Figure S5C, additionally elucidate the effect of the presence of cells and FBS in the in vitro RTgill-W1 assay. Supplemental Data, Figure S5C shows that FBS-containing assays are more capable of retaining C12-BAC in the medium, resulting in more stable systems, particularly for low concentrations, because fractions sorbed to polystyrene and cells are relatively smaller than without FBS. This phenomenon was also observed for phenanthrene with the RTgill-W1 assay in polystyrene well plates [41]. Still, C12-BAC sorption to FBS lowered the bioavailability of the compound. Moreover, Figure 5B clearly shows that the cells can be a significant binding compartment at concentration levels at which cytotoxic effect occurs. This compartment was found to be irrelevant for phenanthrene [41], yet relevant for another largely cationic compound, chlorpromazine, in Caco-2 cell assays [40]. Still, as shown in Figure 5A, as long as freely dissolved concentrations are determined, effect concentrations can be related to bioavailable concentrations in all in vitro assay compositions.

Validation of equilibrium partitioning for the cationic surfactant C12-BAC

The $EC50_{free}$ value, as an alternative parameter mimicking the internal concentration, is regarded as the bioavailable concentration. The present study demonstrates a clear reduction in free concentrations, and therewith bioavailability, of QAC in the presence of sorbent phases in different bioassays. This $EC50_{free}$ in test media with solid suspensions can now be compared with the EC50 in medium-only tests to determine whether the equilibrium partitioning [17] is valid for a particular test chemical and test system.

Figure 2 illustrates that the $EC50_{free}$ of C12-BAC to *D. magna* is independent of the amount of dissolved AHA in 48 h. As summarized in Table 2, the EC50 in medium-only tests of C12-BAC in tests with daphnids, worms, and gill cells are within a factor of 3 of the $EC50_{free}$ in the solid suspensions. The observation that the sorbed fraction does not contribute to toxicity has been widely reported for neutral organic chemicals, both for aquatic organisms in the presence of dissolved HA [39,46] and in in vitro cell assays with different amounts of serum medium [41,47]. These results indicate that the bioavailability of QACs also is strongly inversely related to their sorption affinity to different phases.

Comber et al. [11] suggested that 80% of the very hydrophobic QAC DODMAC was taken up by the worm *L. variegatus* via sediment ingestion [11]. Our study suggests that the QAC C12-BAC is taken up mainly from the aqueous phase or at least that the freely dissolved concentration best represents the chemical activity that drives toxicity. The affinity of C12-BAC to AHA and OECD sediment is high, and the test organisms may have ingested the sediment or taken up dissolved AHA. A study in which particulate organic matter was fed to the daphnid *D. galeata* revealed particulate organic matter in the gut contents, ascertaining that particulate organic matter can be readily ingested by daphnids [27]. Exposure to the high concentrations of bound QAC fractions in our *L. variegatus* and *D. magna* toxicity tests did not show enhanced toxic effects compared with water-only tests. Di Toro et al. [17] concluded that, when equilibrium is reached between the sorbent phase and porewater phase, the pathways of exposure, either to porewater via respiration or to sediment via ingestion, are equivalent. A 24-h equilibrium between C12-BAC and sorbent is sufficient, and such an equilibrated phase is attained. Care has to be taken to ascertain that chemical equilibrium is also reached between the water and the organism in the acute toxicity test, as indicated by our worm study showing that intake of sorbed fractions may enhance the uptake kinetics that lead to an equilibrated system. Based on our data, we suggest the equilibrium partitioning is also valid for this specific cationic surfactant and probably also for other cationic compounds. This implies that toxicity of cationic surfactant in spiked sediment or soil, or the risk of cationic surfactant in natural sediment/soil, can be predicted from sediment- or soil-specific sorption isotherms and aqueous effect concentrations, as suggested by Thomas et al. [12]. Furthermore, it confirms that the presence of dissolved or particulate matter in natural water can substantially attenuate the actual exposure concentrations of cationic surfactants in comparison with total concentrations extracted from the medium, as shown by van Wijk et al. [6]. However, as discussed for cationic surfactants in the European Centre for Ecotoxicology and Toxicology of Chemicals technical report on difficult substances [48], laboratory toxicity tests based on truly dissolved concentrations are of use for environmental risk assessment only if monitored or predicted concentrations in the environment also explicitly and adequately include bioavailability. This includes measurements of passive samplers in situ, as is becoming more common for neutral hydrophobic contaminants [45]; application of accurate sorption models for various environmental substrates (soils, sediments, dissolved colloidal matter), which are still under development [49]; or appropriate bulk-approach toxicity testing in natural water or sediments.

Design of toxicity tests with QACs

Toxic effect levels based on (freely dissolved) bioavailable concentrations are an important condition when comparing the response of different test species to the same chemical or the difference between a series of chemicals in the same toxicity test. Our data show that the adsorptive properties of the C12-BAC can confuse the estimation of actual exposure concentration and the relevant bioavailability in (acute) toxicity tests. The mass balances for C12-BAC show that the freely dissolved fraction can strongly depend on the experimental design, although we still observe large differences in toxic effect concentrations between species based on the bioavailable concentrations (Table 2). The necessity to include the influence of variable QAC sorption affinities on bioavailability applies to several published comparisons between series of analogue QAC structures with increasing alkyl chain length. Findings for series of anionic surfactants demonstrated that surfactants with longer alkyl chains can have stronger interaction with cell membranes and therefore show increased toxicity [50]. Several studies of QACs, however, have shown decreased toxicity with increasing chain

length in acute toxicity studies with *D. magna* and green algae [6,7,14]. *Daphnia* toxicity assays with different polyethoxylated amines showed a lowest nominal effect concentration for the structure with intermediate ethoxylate chain length [51]. In all of these studies, however, effect concentrations were based on nominal concentrations instead of on freely dissolved concentrations. Especially for QACs, the increased adsorption of chemicals with longer alkyl chains to test vessels and sorbents may explain reduced bioavailability and therefore result in apparently lower effect concentrations [6,52,53]. Whether a reduction of QAC toxicity with longer alkyl chains or different head groups still holds true for effect concentrations based on freely dissolved concentrations remains to be verified.

The acute toxicity of several cationic surfactants in different bioassays has been evaluated in earlier work [7,54]. It was found that daphnids tested in 100-mL polystyrene vessels were more than 1 order of magnitude more sensitive to the alkyltrimethylammonium compounds than the fish, tested in 10-L glass aquaria, whereas daphnids showed comparable or lower sensitivity to anionic, zwitterionic, and nonionic surfactants [7]. This confirms the high sensitivity of *D. magna* for cationic surfactant compared with other assays in our study. Sandbacka et al. [7] also found that the 24-h EC50 of alkyltrimethylammonium compounds to gill epithelial cells and hepatocytes isolated from rainbow trout (*Oncorhynchus mykiss*), in 96-well plates and the apparent absence of FBS, were more than 1 order of magnitude higher than the 24-h LC50 to the fish itself. Bernhard and Dyer [54] also found that the effect concentration of hexadecyltrimethylammonium in a 96-well in vitro test was more than an order of magnitude higher than the whole organisms. Using radiolabeling, the latter study did measure dissolved concentrations and showed that the dissolved fraction in medium (free of FBS or other proteins) was approximately 40% to 56%, with 20% to 30% traced in the cell extracted fraction and 14% to 42% unexplained. Although the difference between in vitro and in vivo is not readily explained, Bernard and Dyer [54] showed that the in vitro critical cell residue was in the range of a narcotic mode of action. Applying critical cell residue as a dose metric instead of freely dissolved concentrations may even be the more sensible way of comparing toxicity data from different bioassays and chemicals, for example, to identify why daphnids are the most sensitive test species for cationic surfactants. Although losses to wall sorption with C12-BAC, in clean medium and at concentrations near effect concentrations, appear to have a limited effect on actual effect concentrations of a factor of 2 in the assays in the present study, measuring freely dissolved concentrations should be performed for QACs routinely to verify the extent of losses to test vessels and sorbent phases. The present study demonstrates that commonly used sorbent phases can strongly affect bioavailability and that these sorbed fractions influence effect concentrations for C12-BAC. Even stronger effects of decreased actual exposure concentrations from sorption to walls, cells, and sorbent phases can be expected for more hydrophobic cationic surfactants.

SUPPLEMENTAL DATA

Table S1.
Figures S1–S5. (424 KB DOCX).

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REFERENCES

1. Ying G-G. 2006. Fate, behavior and effects of surfactants and their degradation products in the environment. *Environ Int* 32:417–431.
2. Xia WJ, Onyuksel H. 2000. Mechanistic studies on surfactant-Induced membrane permeability enhancement. *Pharm Res* 17:612–618.
3. Lewis MA. 1991. Chronic and sublethal toxicities of surfactants to aquatic animals: A review and risk assessment. *Water Res* 25:101–113.
4. Buffet-Bataillon S, Tattevin P, Bonnaure-Mallet M, Jolivet-Gougeon A. 2012. Emergence of resistance to antibacterial agents: The role of quaternary ammonium compounds—A critical review. *Int J Antimicrob Agents* 39:381–389.
5. Cserháti T, Forgács E, Oros G. 2002. Biological activity and environmental impact of anionic surfactants. *Environ Int* 28:337–348.
6. van Wijk D, Gyimesi-van den Bos M, Garttener-Arends I, Geurts M, Kamstra J, Thomas P. 2009. Bioavailability and detoxification of cationics: I. Algal toxicity of alkyltrimethyl ammonium salts in the presence of suspended sediment and humic acid. *Chemosphere* 75:303–309.
7. Sandbacka M, Christianson I, Isomaa B. 2000. The acute toxicity of surfactants on fish cells, *Daphnia magna* and fish—A comparative study. *Toxicol In Vitro* 14:61–68.
8. Qv X-Y, Jiang J-G. 2013. Toxicity evaluation of two typical surfactants to *Dunaliella bardawil*, an environmentally tolerant alga. *Environ Toxicol Chem* 32:426–433.
9. Jing G, Zhou Z, Zhuo J. 2012. Quantitative structure–activity relationship (QSAR) study of toxicity of quaternary ammonium compounds on *Chlorella pyrenoidosa* and *Scenedesmus quadricauda*. *Chemosphere* 86:76–82.
10. Ge F, Xu Y, Zhu R, Yu F, Zhu M, Wong M. 2010. Joint action of binary mixtures of cetyltrimethyl ammonium chloride and aromatic hydrocarbons on *Chlorella vulgaris*. *Ecotoxicol Environ Saf* 73:1689–1695.
11. Comber SDW, Rule KL, Conrad AU, Höss S, Webb SF, Marshall S. 2008. Bioaccumulation and toxicity of a cationic surfactant (DODMAC) in sediment dwelling freshwater invertebrates. *Environ Pollut* 153:184–191.
12. Thomas PC, Velthoven K, Geurts M, van Wijk D. 2009. Bioavailability and detoxification of cationics, II. Relationship between toxicity and CEC of cationic surfactants on *Caenorhabditis elegans* (Nematoda) in artificial and natural substrates. *Chemosphere* 75:310–318.
13. Li MH. 2008. Effects of nonionic and ionic surfactants on survival, oxidative stress, and cholinesterase activity of planarian. *Chemosphere* 70:1796–1803.
14. García MT, Ribosa I, Guindulain T, Sánchez-Leal J, Vives-Rego J. 2001. Fate and effect of monoalkyl quaternary ammonium surfactants in the aquatic environment. *Environ Pollut* 111:169–175.
15. Guckert JB, Belanger SE, Barnum JB. 1993. Testing single-species predictions for a cationic surfactant in a stream mesocosm. *Sci Total Environ* 134(Suppl 2):1011–1024.
16. Hermens JLM, Heringa MB, Laak TLT. 2007. Bioavailability in dose and exposure assessment of organic contaminants in (eco)toxicology. *J Toxicol Environ Health A* 70:727–730.
17. Di Toro DM, Zarba CS, Hansen DJ, Berry WJ, Swartz RC, Cowan CE, Pavlou SP, Allen HE, Thomas NA, Paquin PR. 1991. Technical basis for establishing sediment quality for nonionic organic-chemicals using equilibrium partitioning. *Environ Toxicol Chem* 10:1541–1583.
18. Jager T, Fleuren RHLJ, Hogendoorn EA, de Korte G. 2003. Elucidating the routes of exposure for organic chemicals in the earthworm, *Eisenia andrei* (Oligochaeta). *Environ Sci Technol* 37:3399–3404.
19. Rico-Rico A, Temara A, Hermens JLM. 2009. Equilibrium partitioning theory to predict the sediment toxicity of the anionic surfactant C-12-2-LAS to *Corophium volutator*. *Environ Pollut* 157:575–581.
20. Droge STJ, Postma JF, Hermens JLM. 2008. Sediment toxicity of a rapidly biodegrading nonionic surfactant: Comparing the equilibrium partitioning approach with measurements in pore water. *Environ Sci Technol* 42:4215–4221.
21. Mayer P, Vaes WHJ, Wijnker F, Legierse KCHM, Kraaij R, Tolls J, Hermens JLM. 2000. Sensing dissolved sediment porewater concentrations of persistent and bioaccumulative pollutants using disposable solid-phase microextraction fibers. *Environ Sci Technol* 34:5177–5183.
22. Kraaij R, Mayer P, Busser FJM, Van Het Bolscher M, Seinen W, Tolls J, Belfroid AC. 2003. Measured pore-water concentrations make equilibrium partitioning work—A data analysis. *Environ Sci Technol* 37:268–274.

23. Van Der Wal L, Jager T, Fleuren RHLJ, Barendregt A, Sinnige TL, Van Gestel CAM, Hermens JLM. 2004. Solid-phase microextraction to predict bioavailability and accumulation of organic micropollutants in terrestrial organisms after exposure to a field-contaminated soil. *Environ Sci Technol* 38:4842–4848.
24. Jonker MTO, Van Der Heijden SA, Kreitinger JP, Hawthorne SB. 2007. Predicting PAH bioaccumulation and toxicity in earthworms exposed to manufactured gas plant soils with solid-phase microextraction. *Environ Sci Technol* 41:7472–7478.
25. Chen Y, Droge STJ, Hermens JLM. 2012. Analyzing freely dissolved concentrations of cationic surfactant utilizing ion-exchange capability of polyacrylate coated solid-phase microextraction fibers. *J Chromatogr A* 1252:15–22.
26. Chen Y, Hermens JLM, Droge STJ. 2013. Influence of organic matter type and medium composition on the sorption affinity of C12-benzalkonium cation. *Environ Pollut* 179:153–159.
27. Wenzel A, Bergström A-K, Jansson M, Vrede T. 2012. Poor direct exploitation of terrestrial particulate organic material from peat layers by *Daphnia galeata*. *Can J Fisheries Aquat Sci* 69:1870–1880.
28. Organisation for Economic Co-Operation and Development. 2007. Test 225: Sediment-water lumbriculus toxicity test using spiked sediment. *OECD Guidelines for the Testing of Chemicals*. Paris, France.
29. Nederlands Normalisatie Instituut. 1980. *Water: Benodigdheden, Werkwijze en Medium voor het Kweken van Daphnia magna en van de Hiervoor als Voedsel Benodigde Algen*. Delft, The Netherlands.
30. Schirmer K, Chan AGJ, Greenberg BM, Dixon DG, Bols NC. 1997. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. *Toxicol In Vitro* 11:107–119.
31. Organisation for Economic Co-Operation and Development. 2004. Test 202: *Daphnia* sp. acute immobilisation test. *OECD Guidelines for the Testing of Chemicals*. Paris, France.
32. Ralph PJ, Smith RA, MacInnis-Ng CMO, Seery CR. 2007. Use of fluorescence-based ecotoxicological bioassays in monitoring toxicants and pollution in aquatic systems: Review. *Toxicol Environ Chem* 89:589–607.
33. Kramer NI, Busser FJM, Oosterwijk MTT, Schirmer K, Escher BI, Hermens JLM. 2010. Development of a partition-controlled dosing system for cell assays. *Chem Res Toxicol* 23:1806–1814.
34. Tanneberger K, Knöbel M, Busser FJM, Sinnige TL, Hermens JLM, Schirmer K. 2013. Predicting fish acute toxicity using a fish gill cell line-based toxicity assay. *Environ Sci Technol* 47:1110–1119.
35. Davies J, Ward RS, Hodges G, Roberts DW. 2004. Quantitative structure–activity relationship modeling of acute toxicity of quaternary alkylammonium sulfobetaines to *Daphnia magna*. *Environ Toxicol Chem* 23:2111–2115.
36. Colomer A, Pinazo A, García MT, Mitjans M, Vinardell MP, Infante MR, Martínez V, Pérez L. 2012. pH-sensitive surfactants from lysine: Assessment of their cytotoxicity and environmental behavior. *Langmuir* 28:5900–5912.
37. Ferk F, Mišík M, Hoelzl C, Uhl M, Fuerhacker M, Grillitsch B, Parzefall W, Nersesyan A, Mičieta K, Grummt T, Ehrlich V, Knasmüller S. 2007. Benzalkonium chloride (BAC) and dimethyldioctadecyl-ammonium bromide (DDAB), two common quaternary ammonium compounds, cause genotoxic effects in mammalian and plant cells at environmentally relevant concentrations. *Mutagenesis* 22:363–370.
38. Deutschle T, Porkert U, Reiter R, Keck T, Riechelmann H. 2006. In vitro genotoxicity and cytotoxicity of benzalkonium chloride. *Toxicol In Vitro* 20:1472–1477.
39. Day KE. 1991. Effects of dissolved organic carbon on accumulation and acute toxicity of fenvalerate, deltamethrin and cyhalothrin to *Daphnia magna* (Straus). *Environ Toxicol Chem* 10:91–101.
40. Broeders JJW, van Eijkeren JCH, Blaauboer BJ, Hermens JLM. 2012. Transport of chlorpromazine in the Caco-2 cell permeability assay: A kinetic study. *Chem Res Toxicol* 25:1442–1451.
41. Kramer NI, Krismartina M, Rico-Rico A, Blaauboer BJ, Hermens JLM. 2012. Quantifying processes determining the free concentration of phenanthrene in basal cytotoxicity assays. *Chem Res Toxicol* 25:436–445.
42. Gülden M, Mörchel S, Seibert H. 2001. Factors influencing nominal effective concentrations of chemical compounds in vitro: Cell concentration. *Toxicol In Vitro* 15:233–243.
43. Tanneberger K, Knöbel M, Busser FJM, Sinnige TL, Hermens JLM, Schirmer K. 2012. Predicting fish acute toxicity using a fish gill cell line-based toxicity assay. *Environ Sci Technol* 47:1110–1119.
44. Knöbel M, Busser FJM, Rico-Rico A, Kramer NI, Hermens JLM, Hafner C, Tanneberger K, Schirmer K, Scholz S. 2012. Predicting adult fish acute lethality with the zebrafish embryo: Relevance of test duration, endpoints, compound properties, and exposure concentration analysis. *Environ Sci Technol* 46:9690–9700.
45. Lohmann R, Muir D. 2010. Global aquatic passive sampling (AQUA-GAPS): Using passive samplers to monitor POPs in the waters of the world. *Environ Sci Technol* 44:860–864.
46. Freidig AP, Garicano EA, Busser FJM, Hermens JLM. 1998. Estimating impact of humic acid on bioavailability and bioaccumulation of hydrophobic chemicals in guppies using kinetic solid-phase extraction. *Environ Toxicol Chem* 17:998–1004.
47. Heringa MB, Schreurs RHMM, Busser F, Van Der Saag PT, Van Der Burg B, Hermens JLM. 2004. Toward more useful In Vitro toxicity data with measured free concentrations. *Environ Sci Technol* 38:6263–6270.
48. European Centre for Ecotoxicology and Toxicology of Chemicals. 2003. Environmental Risk Assessment of Difficult Substances. Technical Report 088. Brussels, Belgium.
49. Droge STJ, Goss K-U. 2013. Ion-exchange affinity of organic cations to natural organic matter: Influence of amine type and nonionic interactions at two different pHs. *Environ Sci Technol* 47:798–806.
50. Verge C, Moreno A, Bravo J, Berna JL. 2001. Influence of water hardness on the bioavailability and toxicity of linear alkylbenzene sulphonate (LAS). *Chemosphere* 44:1749–1757.
51. Brausch J, Beall B, Smith P. 2007. Acute and sub-lethal toxicity of three POEA surfactant formulations to *Daphnia magna*. *Bull Environ Contam Toxicol* 78:510–514.
52. Li Z, Gallus L. 2007. Adsorption of dodecyl trimethylammonium and hexadecyl trimethylammonium onto kaolinite—Competitive adsorption and chain length effect. *Appl Clay Sci* 35:250–257.
53. Wang J, Han B, Dai M, Yan H, Li Z, Thomas RK. 1999. Effects of chain length and structure of cationic surfactants on the adsorption onto Na-kaolinite. *J Colloid Interface Sci* 213:596–601.
54. Bernhard MJ, Dyer SD. 2005. Fish critical cellular residues for surfactants and surfactant mixtures. *Environ Toxicol Chem* 24:1738–1744.